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14. ABSTRACT

The protocol was approved by the UC Davis (UCD) IRB in September 2011 and approval for the renewal was obtained in July 2012. The protocol is being reviewed by the USAMRMC Office of Research Protections, Human Research Protection Office. We completed an initial site visit with Marinus in October 2011 to discuss drug manufacturing and the protocol. Dr Frank Kooy completed his Year 1 visit in February 2012. He presented on "Towards targeted therapy in the fragile X syndrome using GABAergic drugs". At this meeting, the UCD team and Marinus team, along with Dr Kooy discussed the protocol. We created the electronic database and we are currently testing it. The DSMB was established. We have reviewed our subject database and identified approximately 60 potential subjects for screening. We had a site initiation visit on August 10, 2012 with Marinus. The anticipated drug delivery date to the UCD Investigational Drug Service is August 24, 2012. We continue to have bi-weekly conference calls with Marinus to operationalize the protocol and to prepare for the start of the study once HRPO approves the protocol. Dr Hagerman has also presented on targeted treatments at 5 different conferences during Year 1 of this project.

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INTRODUCTION

This study is a Phase II trial to assess the safety, tolerability, and efficacy of ganaxolone, a $GABA_A$ agonist, for the treatment of behavioral problems including anxiety and inattention in children with FXS. It has been demonstrated in the fragile X mouse model and the Drosophila (fruit fly) model of FXS that the $GABA_A$ system including multiple receptors is dramatically down-regulated. Ganaxolone is drug that enhances a $GABA_A$ activity. We hypothesized that ganaxolone will significantly improve behavioral problems including anxiety, inattention and impulsivity problems in children with fragile X syndrome. We will enroll 60 children, ages 6-17 years, with fragile X syndrome over a 4-year period and they will be randomized to receive either ganaxolone or a placebo initially and then crossed over after 6 weeks. We will use innovative outcome measures in addition to standard outcome measures that have been successful in previous treatment trials in fragile X syndrome at baseline and follow-up visits.

BODY

The components of TASK 1 (Administrative study set-up) were completed from July 2011 to September 2012. In August 2011, the contract between the Department of Defense (DOD) and the University of California at Davis (UCD) was executed. From August 2011 through January 2012, the Principal Investigator (PI), Dr. Randi Hagerman, and Co-PIs, Dr. David Hessl and Dr. Michael Rogawski, worked with Marinus to establish a subcontract where intellectual property and a material plan was created and agreed upon. This task was completed upon contract execution between University of California, Davis (UCD) and Marinus.

The UCD Institutional Review Board (IRB) protocol approval was obtained in August 2011 and then renewed in July 2012. We are currently working with U.S. Army Medical Department Medical Research and Material Command (USAMRMC) Human Research Protection Office (HRPO) for protocol approval and we anticipate receiving approval by September 2012. No subjects have been screened for the trial because we need the HRPO approval before screening begins, however we have identified approximately 60 potential patients to be screened as soon as the HRPO approval is obtained to help catch up with the enrollment timeline.

Dr. Frank Kooy, consultant on the project, presented in February 2012 with new animal data that shows efficacy of ganaxolone in the knock-out mouse model for the seizures. Please see Heulens et al., 2012 which is attached in the Appendix.

In July 2012, Dr. Hagerman, Dr. Hessl, and Dr. Rogawski along with the Chief Regulatory Officer of Marinus, Dr. Gail Farfel, finalized the operational version of the protocol. The Federal Drug Agency (FDA) Form 1572 and the Investigational New Drug (IND) were filed with the Division of Neurology at the FDA.

TASK 2 (Manufacturing and distribution of clinical study drug) was completed in August 2012. Marinus initiated the purchase of raw materials and secured manufacturing space to produce clinical supplies for this trial. The active pharmaceutical ingredient, the formulation of the active drug and placebo, the validation testing and release, the bottling, labeling, and storage were completed between February to August 2012. The drug and placebo are anticipated to be delivered to the UCD Investigational Drug Services by August 24, 2012.

The components of TASK 3 (Study initiation) have almost been completed. We completed a site initiation visit between UCD and Marinus in October 2011. The protocol measures have been compiled and the eye-tracking protocol, event related potential paradigm, and Test of Attentional Performance for Children have been tested and are ready for use. The biomarker protocol for mTOR dysregulation including S6 Kinase and EIF4E (Eukaryotic translation initiation factor 4E), is finalized and Dr. Flora Tassone and her lab are ready to receive molecular samples. Please see Hoeffer et al., 2012 which is attached in the Appendix. The electronic database, along with the electronic case report forms, were created in REDCap (Research Electronic Data Capture). Currently the database is being tested. The Data Safety Monitoring Board (DSMB) has been created and the individuals are Robin Hansen, M.D. (clinician), Mary Beth Steinfeld, M.D. (clinician), and Danielle Harvey, Ph.D. (statistician).

The last component of TASK 3, recruitment, has not begun since we have not obtained USAMRMC HRPO approval for human subjects research. However, we have identified approximately 60 potential subjects. Once USAMRMC HRPO approval has been received, we will begin to contact these 60 potential subjects to see if there is interest in participating in the trial and to determine eligibility. We anticipate beginning in September 2012.

TASK 4, the last Task of the statement of work, which was supposed to begin in months 9-12, has not been initiated. There have been no subjects consented or screened. In Year 1, we were supposed to enroll 10 subjects, but because we have not received HRPO approval of the protocol or the drug, we are unable to meet this goal. Safety Review and data-entry also has not taken place since there are no active subjects. To catch up with the enrollment timeline, we plan to enroll 30 subjects in Year 2 instead of the initial planned 20 subjects. We anticipate beginning enrollment and randomization to drug or placebo to begin in September 2012.

KEY RESEARCH ACCOMPLISHMENTS

There are no key research accomplishments to report since subjects have not been enrolled yet.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

At this early date we do not have reportable outcomes.

CONCLUSION

We have obtained UC Davis (UCD) IRB approval for the protocol and we are in the process of obtaining USAMRMC HRPO approval for the protocol. We completed an initial site visit between UCD and Marinus in October 2011. Dr Frank Kooy, consultant on the project, presented in February 2012 with new animal data that shows efficacy of ganaxolone in the KO mouse model for the seizures. The DSMB was established and the electronic database was created and is currently being tested. The site initiation visit was completed between UCD and Marinus in August 2012. We anticipate drug to be delivered to the UCD Investigational Drug Service by August 24, 2012. Screening and enrollment of subjects will begin once USAMRMC HRPO approval has been obtained, which is anticipated to be done by September 2012. We are planning to make up the delay in getting the ganaxolone for timely dosing and in getting the HRPO approval by seeing 30 patients (10 originally planned for the first year and 20 for the second year) in this second year for the study which will get us back on the original timeline of patient visits. If we can demonstrate efficacy of ganaxolone in our outcome measures this will have a major impact on the treatment of fragile X syndrome and move us closer to a cure for these children.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science, Military Medicine,* etc.).

1. C. A. Hoeffer *et al.*, *Genes, brain, and behavior* **11**, 332 (Apr, 2012).

2. I. Heulens, C. D'Hulst, D. Van Dam, P. P. De Deyn, R. F. Kooy, *Behavioural brain research* **229**, 244 (Apr 1, 2012).

APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Please find attached the following journal articles.

1. C. A. Hoeffer *et al.*, *Genes, brain, and behavior* **11**, 332 (Apr, 2012).

2. I. Heulens, C. D'Hulst, D. Van Dam, P. P. De Deyn, R. F. Kooy, *Behavioural brain research* **229**, 244 (Apr 1, 2012).

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Altered mTOR signaling and enhanced CYFIP2 expression levels in subjects with fragile X syndrome

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Fragile X syndrome (FXS) is the most common form of inherited intellectual disability and autism. The protein (FMRP) encoded by the fragile X mental retardation gene (*FMR1*), is an RNA-binding protein linked to translational control. Recently, in the *Fmr1* knockout mouse model of FXS, dysregulated translation initiation signaling was observed. To investigate whether an altered signaling was also a feature of subjects with FXS compared to typical developing controls, we isolated total RNA and translational control proteins from lymphocytes of subjects from both groups (38 FXS and 14 TD). Although we did not observe any difference in the expression level of messenger RNAs (mRNAs) for translational initiation control proteins isolated from participant with FXS, we found increased phosphorylation of the mammalian target of rapamycin (mTOR) substrate, p70 ribosomal subunit 6 kinase1 (S6K1) and of the mTOR regulator, the serine/threonine protein kinase (Akt), in their protein lysates. In addition, we observed increased phosphorylation of the cap binding protein eukaryotic initiation factor 4E (eIF4E) suggesting that protein synthesis is upregulated in FXS. Similar to the findings in lymphocytes, we observed increased phosphorylation of S6K1 in brain tissue from patients with FXS ($n = 4$) compared to normal age-matched controls ($n = 4$). Finally, we detected increased expression of the cytoplasmic FMR1-interacting protein 2 (CYFIP2), a known FMRP interactor. This data verify and extend previous findings using lymphocytes for studies of neuropsychiatric disorders and provide evidence that misregulation of mTOR signaling observed in the FXS mouse model also occurs in human FXS and may provide useful biomarkers for designing targeted treatments in FXS.

Keywords: CYFIP1, CYFIP2, fragile X, mTOR, phosphorylation

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Fragile X syndrome (FXS) is the most common inherited cause of mental retardation, and the most common single gene mutation associated with autism (Demark *et al*. 2003; Hatton *et al*. 2006; Jacquemont *et al*. 2007; Kaufmann *et al*. 2004; Loesch *et al*. 2007; Rogers *et al*. 2004). It is caused by a trinucleotide repeat expansion $(CGG)_n$ in the 5′ untranslated region of the fragile X mental retardation 1 gene (*FMR1*) located at Xq27.3. The full mutation, present in individuals having more than 200 CGG repeats, typically involves methylation and subsequent transcriptional silencing of the *FMR1* gene, resulting in diminished or absent production of the *FMR1* protein, FMRP (Fu *et al*. 1991; Pieretti *et al*. 1991; Verkerk *et al*. 1991; Yu *et al*. 1991). Loss of FMRP results in aberrant brain development and function (Bagni & Greenough 2005).

It was recently reported that the mammalian target of rapamycin (mTOR) signaling is upregulated in a mouse model of FXS (Sharma *et al*. 2010). The loss of FMRP resulted in enhanced mTOR signaling that was associated with increased formation of the eukaryotic initiation factor complex 4F (eIF4F). These findings suggest that in addition to its RNA-binding activity, FMRP also plays a role in the regulation of translation initiation and subsequent protein synthesis. Whether this is the case in individuals with FXS is unknown.

Although FXS is associated with a characteristic phenotype, there is considerable within-syndrome variation in the severity of the phenotype and the profile of impairments, with the most interesting being comorbidity with autism. From the most recent studies, the prevalence of autism spectrum disorder (ASD) is approximately 60% in individuals with FXS (Harris *et al*. 2008). A subgroup of patients with FXS also presents with a Prader-Willi-like phenotype (PWP), which includes severe hyperphagia, obesity, hypogonadism and autism. Prader-Willi syndrome (PWS) is an obesity syndrome resulting from a loss of paternally derived genes at 15q11-13, which regulate metabolism or energy homeostasis. The PWP of FXS was first reported in males with extreme obesity, short stature, short fingers and toes and hypogonadism (de Vries *et al*. 1993; de Vries & Niermeijer 1994; Fryns *et al*. 1987; Hagerman & Hagerman 2002; Schrander-Stumpel *et al*. 1994) but the patients do not show genetic

abnormalities at 15q11-13, which normally is associated with PWS. Interestingly, very high rates of autism are observed in patients with FXS and the PWP (Nowicki *et al*. 2007).

The findings of an upregulation of the mTOR signaling in the FXS mouse model, combined with the very high rates of autism associated with FXS with and without the PWP (Nowicki *et al*. 2007), prompted us to investigate mTOR signaling in subjects with FXS and FXS with PWP. Because the molecular signaling effects resulting from FMRP loss are likely causal in the wide-ranging severity of FXS symptoms, including autism, identifying the effects of FMRP loss on molecular signaling pathways, like those governing translation, are key to advancing our ability to treat the disorder.

Methods

Study participants

Fifty-two subjects (49 males, 3 females) were included in the study, except for the cytoplasmic FMR1 interacting protein 1 *(CYFIP1)* messenger RNA (mRNA) measurements, which comprised an additional 30 subjects. Participants were recruited through the Fragile X Research and Treatment Center at the UC Davis MIND Institute in Sacramento (CA) and included a total of 38 cases with FXS, 10 of which were mosaics (both methylation and size mosaics) (mean 19 ± 2 years, range 4–68 years). Seven patients had FXS without ASD, while 31 participants presented with both FXS and ASD. Fourteen subjects also had the PWP and 12 of them had ASD. Fourteen typically developing (TD) controls (ranging from 21 to 40 CGG repeats) (mean age 26 ± 5 years, range 2–55 years) were also included in the study. This study was approved by the Institutional Review Board of the University of California.

Clinical evaluation and assessment measures for autism

A complete medical evaluation, including medical history, psychological testing and physical examination was conducted on each subject including controls. Individuals were confirmed to have ASD by a multidisciplinary assessment. This assessment included the Autism Diagnostic Interview-Revised (ADI-R) (Rutter *et al*. 2003a) which is a standardized, semi-structured, investigator-based interview for caregivers of individuals with autism or pervasive developmental disorders, and the Autism Diagnostic Observation Schedules (ADOS) (Lord *et al*. 1999) which is a semi-structured, standardized assessment of the child in which the researcher observes the social interaction, communication, play and imaginative use of materials for children suspected of having ASD. The DSM IV-TR (APA 2000) criteria for ASD, was also applied and, a team consensus lead to a diagnosis of either autism, pervasive developmental disorders not otherwise specified (PDD-NOS) or no ASD. In this study, the autism and PDD-NOS diagnoses were collapsed to ASD.

Cognitive and adaptive measures

The Wechsler Scale including the WISC-IV (Wechsler 2003), WPPSI-III (Wechsler 2002) or WASI (Wechsler 1999) (where age appropriate) were used for all patients with FXS for assessing IQ. Controls were screened for ASD traits using Social Communication Questionnaire (SCQ) (Rutter *et al*. 2003b) and for developmental delay with the Vineland Adaptative Behavior Scale (VABS) (Sparrow *et al*. 2005). Only those who scored within the normal range were included in this study.

Molecular measures

DNA analysis

To confirm the presence of FXS, a blood sample was obtained from each subject for the measurement of the CGG repeat number in the *FMR1* gene. Genomic DNA was isolated from peripheral blood leukocytes using standard methods (Puregene Kit; Gentra Inc., Minneapolis, MN, USA). For Southern blot analysis, 5–10 μg of isolated genomic DNA was digested with EcoRI and NruI. Probe hybridization used the *FMR1*-specific dig-labelled StB12.3. Details were as previously described (Tassone *et al*. 2008). Polymerase chain reaction (PCR) analysis was performed on all the subjects using primer c and f as described in Filipovic-Sadic *et al*. (2010) and Tassone *et al*. (2008). Analysis and measurement of trinucleotide allele size, as well as the determination of the methylation status were determined using an Alpha Innotech Fluor Chem 8800 Image Detection System (Alpha Innotech, San Leandro, CA, USA) and the ABI 3730XL 96-Capillary Electrophoresis Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).

Brain tissues

Brain tissue derived from four males with FXS were from autopsies, performed in accordance with University of California, Davis, Institutional Review Board (IRB) approved protocols. One hemisphere of the fresh brain was cut into 1-cm coronal sections and frozen at −80°C and protein extracts and total RNA were isolated from a frozen 0.5-cm section. Case history and clinical/molecular details of the subjects are as described in Greco *et al*. (2011) and Hunsaker *et al*. (2011). Age-matched control samples were taken from four neurologically normal male cases that were obtained from the autopsy tissue repository at the University of California, Davis Medical Center Department of Pathology and from the Maryland Brain Bank (Table 1).

Measurement of gene expression levels

Total RNA was isolated from Tempus tubes using the ABI PRISM™ 6100 Nucleic Acid PrepStation per the manufacturer's protocol (Applied Biosystems). Total RNA was isolated from brain tissue using standard procedures (Trizol, Invitrogen, Grand Island, NY, USA). Expression levels of the *FMR1* gene and of those genes involved in the mTOR pathway cascade were measured by real-time quantitative PCR fluorescence (QRT-PCR) method using primers and probe specific for each single gene (Assay on Demand, Applied Biosystems). Details of the method and its application to the study of *FMR1* mRNAs are as described in Tassone *et al*. (2000).

Lymphocyte extraction and storage

Approximately 6 ml of whole blood from subjects was collected into BD Vacutainer™ CPT™ tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) containing heparin, for isolation of white cells. Lymphocytes were separated, aliquoted (~2×10^6 cells for cryovial), and stored in liquid nitrogen within 24 h of collection according to manufacturer's

Table 1: Summary of the pathology of the post-mortem brain tissue of the eight cases described in this study

Subject	Category	Age at death (years)	PMI (h)	Cause of death
Case 1	FXS	23	16	Cardiac arrest
Case 2	FXS	57	20	Choking on food
Case 3	FXS	64	12	Liver neoplasm
Case 4	FXS	74	40	Pulmonary disease and abdominal complications
Control 1	Normal	20	36	Gun shot
Control 2	Normal	57	16	Accident, multiple injuries
Control 3	Normal	68	17	Cardiac arrest
Control 4	Normal	88	11	Cardiac arrest

FXS, fragile X syndrome; PMI, post-mortem interval.

directions, until use. Lymphocytes were maintained under these storage conditions until used for extractions.

Protein extraction and Western blotting

Cells were spun down at 17 000 ***g*** for 10 min and washed twice in wash buffer [150 mM NaCl, 50 mM Tris, 2 mM ethylenediaminetetraacetic acid (EDTA)], then added to homogenization buffer containing protease and phosphatase inhibitors. Cells were briefly sonicated on ice (~10 seconds each) in brief pulses (2–3 seconds/pulse). Nucleic acids in homogenate was then sheared using a sterile 21 gauge syringe thrice. Lysed cell slurry was cleared at 17 000 ***g*** at 4°C then quantified using Bradford technique (Pierce, Rockford, IL, USA). Protein concentrations were determined by absorbance reading at 562λ (Biotek Synergy 2 Plate reader, Winooski, VT, USA). A measure of 30 μg of total protein was combined with 6 × sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) buffer (final SDS 1%). Samples were heated at 95°C for 5 min and snap chilled before loading. Proteins were separated on Novex 4–12% gradient Tris-Bis gels (Invitrogen) then transferred to PVDF blots using conventional methodology. Blots were blocked in 0.2% I-Block (Tropix, Carlsbad, CA, USA), and then incubated overnight with primaries at 4°C. Bands were resolved using HRP conjugated secondary and visualized using ECL+ (GE-Amersham, Waukesha, WI, USA) on a KODAK 4000MM (Carestream, Rochester, NY, USA) or G.E. LAS4000 (Piscataway, NJ, USA) imaging system. All chemiluminescent signals were obtained in the linear range of detection as confirmed by time–course of exposures and saturation detection (G.E. LAS4000). Blots were subsequently stripped and reprobed with total antibody. Samples that failed to generate western signals that detected glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or generated protein of interest signals less than 10% of background were excluded from analyses.

Antibodies

All antibodies used in this study except for CYFIP1 were commercially obtained. Primaries: total mTOR (Bethyl Labs, Montgomery, TX, USA) 1:2000, phospho Ser 2448 mTOR (Cell Signaling, Danvers, MA, USA) 1:1000, phospho Threonine 389 p70 (Millipore, Billerica, MA, USA) 1:1000, total p70 (Cell Signaling), phospho-Ser235/236 S6 (Bethyl Labs) 1:2000, phospho Serine 209 eIF4E (Cell Signaling) 1:1000, total eIF4E (Bethyl Labs) 1:2000, phospho Serine 473 Akt (Cell Signaling) 1:1000, pan Akt (Cell Signaling) 1:1000, phospho Thr202/Tyr204 p44/42 (Erk1/2) (Cell Signaling) 1:3000, p44/42 MAPK (Erk1/2) rabbit monoclonal (Bethyl Labs) 1:3000, CYFIP1 1:1000, CYFIP2 (Millipore) 1:1000, GAPDH (Novus, St. Charles, MO, USA) 1:10 000; Secondaries: Goat anti-rabbit-HRP (Promega, Madison, WI, USA) 1:5000, Goat anti-mouse-HRP (Promega) 1:5000.

Statistical analysis

Statistical analysis comparison of mRNA expression [*CYFIP2, FMR1,* Janus kinase and microtubule interacting protein 1 (*JAKMIP1*), *RPS6KB1, RPS6KB2, mTOR, Akt, EIF4EBP1* and G protein-coupled receptor 155 (*GPR155*)] was based on analysis of variance after standard descriptive and graphical analyses. Analysis comparing protein (p389/p70, p209/EIF4E, CYFIP2 and pmTOR/mTOR) collapsed over groups (e.g. FXS vs. control) was based on the standard *t*-test; if equal variance hypothesis was rejected, then Satterthwaite Two Sample *t*-test was applied. All statistical tests are two-tailed at significance level 0.05. Statistical analysis was performed in SAS version 9.2.

Results

Measurements of mRNA expression levels in lymphocytes from patients with FXS

Gene expression levels were measured by QRT-PCR on total RNA isolated from peripheral blood leukocytes derived from subjects with FXS with and without the PWP and from controls. As expected, we found substantial reduction in the relative amounts of *FMR1* mRNA expression levels between FXS (with and without PWP) and normal groups, with an ~75% reduction in total normal signal (FXS = 0.368, SD 0.573; normal= 1.459, SD 0.244; df = 46, $t = 9.49$, $P < 0.01$). Detectable *FMR1* mRNA was observed in FXS mosaics, which carry unmethylated, transcriptionally active, expanded alleles.

Consistent with our previous findings we found a reduction in *CYFIP1* mRNA expression levels, which encodes an FMRP binding protein and is a repressor of eIF4E activity (Napoli *et al*. 2008; Schenck *et al*. 2003), in the blood of patients with FXS and PWP (df = 78, $t = 3.14$, $P = 0.04$) compared to normal controls (Nowicki *et al*. 2007) (Table 2). A recent study reported an increase in *CYFIP1* expression in lymphoblastoid cells isolated from patients with autism (Nishimura *et al*. 2007). They also reported on an increased expression of JAKMIP1 and of GPR155 in both lymphoblastoid cell lines derived from subjects with autism and in the brains of *Fmr1* knockout mice (Nishimura *et al*. 2007). Interestingly, we did not observe a change in the mRNA expression for these two genes in our study (Table 2). Using QRT-PCR, we also measured the mRNA expression levels of *CYFIP2*, the *CYFIP1* paralog, and of the translational control elements, including S6K1, S6K2, mTOR, Akt and eIF4E-binding protein (4E-BP) in patients with FXS compared to typical developing controls. However, no significant differences were observed (Table 2) indicating that in lymphocytes, it is unlikely that FMRP exerts control over the abundance or stability of mRNAs encoding regulators of translational initiation. Gene expression levels were also measured in brain tissue from subjects with FXS and controls. Although, as expected, *FMR1* mRNA expression levels in the brain were significantly different between FXS and controls (FXS = 0.012, SD = 0.017; normal = 0.631, SD = 0.192; df = 3, $t = 6.42$, $P < 0.01$ in frontal cortex and FXS = 0.015, SD = 0.009; normal = 0.463, SD = 0.166; df = 3, $t = 5.05$, $P = 0.01$ in cerebellum) the expression levels of mRNAs for translational initiation control proteins were similar in the two groups.

Phosphorylation of substrates and regulators of mTOR signaling is increased in patients with FXS

One of the most well-described mTOR substrates is p70 S6K1 (S6K1), which regulates a number of activities related to translation initiation including ribosomal maturation and RNA helicase activity. The activity of S6K1 is regulated by phosphorylation at multiple sites (Jacinto & Lorberg 2008) with Threonine 389 (T389) being the site of mTOR-dependent regulation (Burnett *et al*. 1998; Klann & Dever 2004). Because mTOR is known to be dysregulated in mouse models of FXS (Sharma *et al*. 2010) we examined regulation of this site in S6K1 isolated from lymphocytes of human subjects. We found that compared to normal controls, phosphorylation at T389 (pT389) was enhanced in individuals with FXS. The ratio of levels of pT389 S6K1/Total S6K1 (pS6K1/S6K1) for individuals with FXS compared to normal individuals was (pS6K1/s6K1 ratio: FXS mean = 1.885, SD = 1.463; normal = 1.062, SD = 0.233; df = 30, $t = 2.90$, $P < 0.01$; Fig. 1a,b). A subset of our patients with FXS and normal

Table 2: mRNA expression levels in the blood of patients with FXS and PWP compared to controls

	Full mutation with PWP (A)			Full mutation w/o PWP (B)		Normal Control (C)			A vs C			A vs B			B vs C		
mRNA	*N*	Mean*	SD	*N*	Mean*	SD	*N*	Mean*	SD	df	*t*	*P*	df	*t*	*P*	df	*t*	*P*
FMR1	15	0.383	0.428	23	0.358	0.659	13	1.459	0.244	48	−5.49	<0.01	48	0.14	0.89	48	−6.13	<0.01
CYFIP1	23	0.423	0.256	37	0.532	0.162	21	0.616	0.204	78	−3.14	<0.01	78	−2.02	0.05	78	−1.5	0.14
CYFIP2	9	0.912	0.134	20	0.963	0.207	10	0.867	0.225	36	0.49	0.62	36	−0.63	0.53	36	1.24	0.22
RPS6KB1	15	1.128	0.198	23	1.151	0.194	13	1.153	0.233	48	−0.32	0.75	48	−0.33	0.74	48	−0.02	0.98
RPS6KB2	15	1.274	0.451	23	1.26	0.224	13	1.074	0.374	48	1.55	0.13	48	0.13	0.90	48	1.57	0.12
mTOR	15	1.138	0.293	23	1.206	0.285	12	1.037	0.312	47	0.89	0.38	47	−0.7	0.49	47	1.62	0.11
Akt	15	1.035	0.201	23	1.103	0.122	13	1.426	0.179	48	−0.29	0.69	48	−0.3	0.73	48	−0.1	0.10
EIF4EBP1	15	0.4	0.108	23	0.377	0.082	12	0.422	0.138	47	−0.53	0.60	47	0.66	0.51	47	−1.19	0.24
GPR155	15	12.21	7.132	23	13.784	4.344	13	11.408	5.238	48	0.38	0.70	48	−0.86	0.39	48	1.24	0.22
JAKMIP1	15	0.597	0.293	23	0.596	0.365	13	0.695	0.381	48	−0.75	0.46	48	0.01	0.99	48	−0.82	0.42

Akt, Protein Kinase B; CYFIP1 , Cytoplasmic FMR1-interacting protein 1; CYFIP2, Cytoplasmic FMR1-interacting protein 2; EIF4EBP1, Eukaryotic translation initiation factor 4E-binding protein 1; FMR1, fragile X mental retardation 1; GPR155, G protein-coupled receptor 155; JAKMIP1, janus kinase and microtubule interacting protein 1; mTOR, mammalian target of rapamycin; PWP, Prader Willi-like phenotype; RPS6KB1, Ribosomal protein S6 kinase, 70 kDa, polypeptide 1; RPS6KB2, Ribosomal protein S6 kinase, 70 kDa, polypeptide 2.
*Gene expression levels.

Figure 1: Quantification of phospho-proteins expression levels and representative Western blots in peripheral blood leukocytes of controls and subjects with FXS. (a) Patients with FXS ($n = 28$) display increased levels of phosphorylated Threonine 389 (pT389) p70 S6 kinase 1 (S6K1) and (pS235/236) S6 compared to normal controls ($n = 14$, $P = 0.0069$). (b) Representative Western blot images for pT389 P70 S6K, P70 S6K, pS235/236 S6, GAPDH (loading control) from lysates from four patient sets. (c) Patients with FXS ($n = 27$) show no statistically significant difference in the levels of phosphorylated Serine 2448 (pS2448) mammalian target of rapamycin (mTOR) compared to normal controls ($n = 14$). (d) Representative Western blot images for pS2448 mTOR, mTOR, GAPDH (loading control, same blot as above) from lysates from four patient sets. (e) Patients with FXS ($n = 9$) show increased levels of phosphorylated Serine 473 (pS473) AKT (PKB) kinase compared to normal controls ($n = 7$, $P = 0.0354$). (f) Representative Western blot images for pS473 AKT, AKT, from lysates from four patient sets. The percent (%) of phospho-signal were normalized to total protein signal for each graph. The error bars represent standard error in each graph. Blots were checked for efficient stripping prior to reprobing.

controls were tested for the activation of S6K1 substrates. Consistent with our S6K1 observations, we also observed increased phosphorylation levels of the S6K1 substrate, ribosomal protein S6 at serine 235/236 (pS6/GAPDH ratio: FXS mean = 1.414, SD = 0.358, normal mean = 0.638, SD 0.275; df = 7, $t = 4.72$, $P < 0.01$; Fig. 1b). No difference in the total amount of protein expression was observed. Surprisingly, when we examined phosphorylation of S2448 on mTOR, a site of multiple kinase activity including S6K1 (Sharma *et al*. 2010) we found no significant difference between FXS and normal individuals in the ratio of levels of phospho/total mTOR (pmTOR/mTOR) (Fig. 1c,d). For individuals with FXS

compared to normal individuals this ratio was (pmTOR/mTOR ratio: FXS = 1.225, SD = 0.794; normal = 0.961, SD = 0.390; df = 39, $t = -1.43$, $P = 0.16$). Sharma *et al*. (2010) also reported increased levels of phosphorylation of Akt, an upstream activator of mTOR, at Serine 473 (S473) in FMRP KO mouse brains. This regulatory site is conserved in humans; therefore we also examined S473 phosphorylation levels in FXS tissues (Fig. 1e). Consistent with Sharma *et al*. (2010) and with our data (Fig. 1a,b) we also observed elevated S473 expression levels in FXS lymphocytes (Fig. 1e,f) (pS473 Akt/Tot Akt ratio: FXS mean = 0.177, SD = 0.030; normal mean = 0.076, SD = 0.029; df = 13, $t = 2.34$, $P = 0.04$).

The finding that phosphorylation at T389 of S6K was enhanced in patients with FXS prompted us to examine the phosphorylation levels of eIF4E, another critical regulator of cap-dependent translation. eIF4E encodes m7-GTP cap binding activity, providing a physical link between substrate mRNA and the translational initiation machinery. Phosphorylation at serine 209 (pS209) of eIF4E is correlated with increased translation (Flynn & Proud 1995; Gingras *et al*. 1999; Mckendrick *et al*. 1999). Similar to S6K1, we found robust increases in the ratio of levels of pS209 eIF4E/total eIF4E (p4E/4E) in patients with FXS (Fig. 2a,b) while the total amount of eIF4E protein was unaltered. The ratio

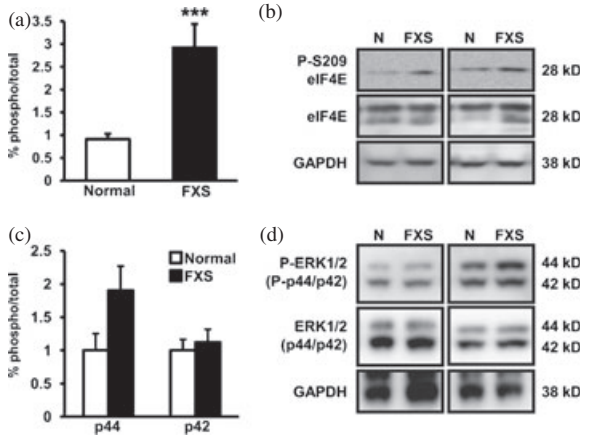

Figure 2: Quantification of phospho-proteins expression levels and representative Western blots in peripheral blood leukocytes of controls and subjects with FXS. (a) Patients with FXS ($n = 20$) display increased levels of phosphorylated Serine 209 (pS209) eukaryotic initiation factor 4E (eIF4E) compared to normal controls ($n = 14$, $P = 0.0006$). (b) Representative Western blot images for pS2098 eIF4E, eIF4E, GAPDH (loading control) from lysates from four patient sets. (c) Patients with FXS ($n = 13$) show no difference in phospho Threonine 202/Tyrosine 204 ERK1(p44)/ERK2(p42) levels compared to normal controls ($n = 10$). We observed a trend for increased pERK1/ERK2 in FXS patients but the difference was not significant ($P = 0.0989$). (d) Representative Western blot images for pT202/Y204 ERK1/2, total ERK1/2, and GAPDH (loading control) from lysates from four patient sets. The percent (%) of phospho-signal was normalized to total protein signal for each graph. The error bars represent standard error in each graph. Blots were checked for efficient stripping prior to reprobing.

of levels of p4E/4E for individuals with FXS compared to normal individuals was (p4E/4E ratio: FXS mean = 2.923, SD = 2.690; normal mean = 0.912, SD = 0.447; df = 30, $t = 3.85$, $P < 0.01$). eIF4E is regulated by the activity of the extracellular-signal regulated kinase (p44)1 and (p42)2 (ERK 1/2) (Waskiewicz *et al*. 1999) and levels of phosphorylation of ERK1/2 at Threonine 202/Tyrosine 204 (pERK1/2) have been shown to be elevated in FXS model mice (Hou *et al*. 2006). Thus, we investigated the possibility that pERK1/2 levels were also elevated in FXS lymphocytes. Although we found a trend for increased levels of pERK in FXS lymphocytes in the larger isoform (ERK1) (Fig. 2c,d), the difference was not statistically significant (pERK1/ERK1: $P = 0.10$; pERK2/ERK2: $P = 0.10$) (Fig. 2c). These results, combined with the increased levels of phosphorylation of T389 on S6K1 (Fig. 1a), suggest that the mTOR but not ERK1/2 signaling dysregulation observed in *Fmr1* KO mice also is present in individuals with FXS and provides evidence of increased translational activity in lymphocytes of subjects with FXS.

CYFIP1 protein levels are normal but CYFIP2 protein expression is increased in patients with FXS

Because we found markers for signaling consistent with enhanced translation and decreased *CYFIP1* mRNA levels in FXS patients, we chose to examine the RNA and protein expression levels of *CYFIP1* and *CYFIP2* (Schenck *et al*. 2001). Surprisingly, when we examined CYFIP1 protein levels in protein lysates obtained from lymphocytes we detected no difference in the levels of CYFIP1 protein between FXS patients and normal controls (Fig. 3a,b) (CYFIP1/GAPDH ratio: FXS mean = 0.524, SD = 0.249; normal mean = 0.386, SD = 0.214; df = 15, $t = 1.21$, $P = 0.24$).

Consistent with the notion that FMRP acts as suppressor of *CYFIP2* expression by sequestration of its mRNA (Schenck *et al*. 2001); we found elevated expression levels of CYFIP2 in the lymphocytes of FXS when compared to normal controls (Fig. 3c,d). The ratio of CYFIP2/GAPDH for individuals with FXS compared to normal individuals was (CYFIP2/GAPDH: FXS mean = 2.094, SD = 1.195; normal mean = 1.118, SD = 0.553; df = 39, $t = -3.57$, $P < 0.01$). This increase was specific to CYFIP2 protein as total levels of GAPDH, mTOR and S6K1 (Figs 1b,d and 3d) were unchanged in FXS. Interestingly, in contrast to *CYFIP1* mRNA expression levels, this increase was observed without a detectable change in *CYFIP2* mRNA expression levels (Table 2) suggesting that *CYFIP2* expression in the blood is normally limited by the availability of its mRNA for translation and not by increases in transcription.

Phosphorylation of p70 S6K1 is increased in brains of patients with FXS

Because FXS defines a series of symptoms that includes intellectual disabilities, increased anxiety, mild to severe cognitive impairment and comorbidity with autism (Loesch *et al*. 2007; Rogers *et al*. 2001) we sought to explore if our findings in fresh lymphocytes could be extended to brain tissue from FXS individuals. We obtained tissue from the cerebellum and frontal lobes of four patients

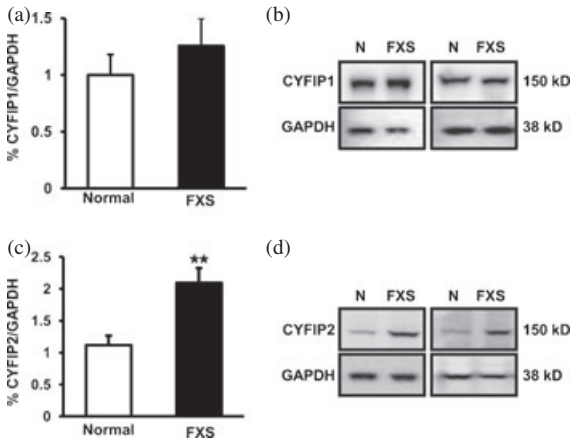

Figure 3: Quantification of phospho-proteins expression levels and representative Western blots in peripheral blood leukocytes of controls and subjects with FXS. (a) Patients with FXS ($n = 6$) display no difference in levels of FMRP interacting protein, CYFIP1, compared to normal controls ($n = 9$). (b) Representative Western blot images for CYFIP1, GAPDH (loading control) from lysates from four patient sets. (c) Patients with FXS ($n = 27$) display increased levels of FMRP interacting protein, CYFIP2, compared to normal controls ($n = 16$, $P = 0.0010$). (d) Representative Western blot images for CYFIP2, GAPDH (loading control) from lysates from four patient sets. The percent (%) of CYFIP1 or CYFIP2 were normalized to total GAPDH protein signal for each graph. The error bars represent standard error in each graph.

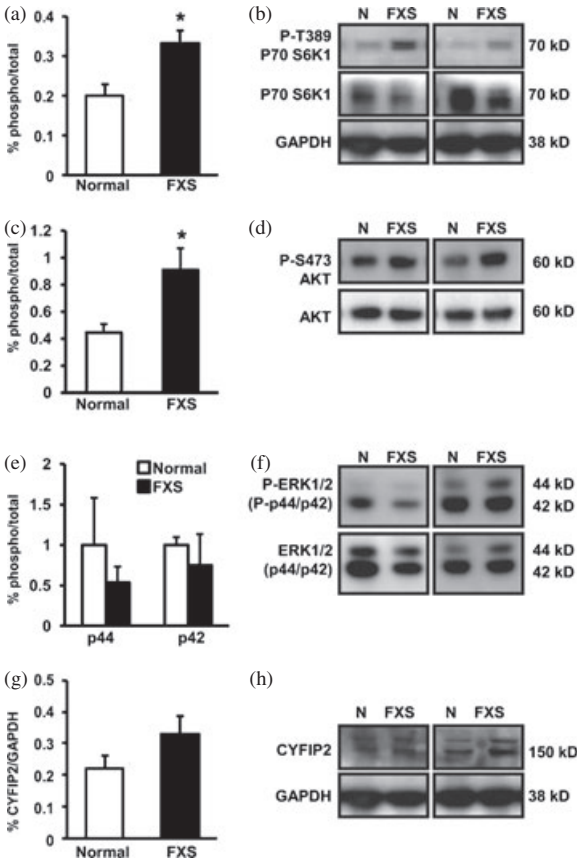

Figure 4: Quantification of phospho-proteins expression levels and representative Western blots in brain tissue from controls and subjects with FXS. (a) Patients with FXS ($n = 4$) display increased levels of phosphorylated Threonine 389 (T389) p70 S6 kinase 1 (S6K1) compared to normal controls ($n = 4$, $P = 0.0274$). The percent (%) phospho-signal normalized to total protein signal for each graph. (b) Representative Western blot images for pT389 P70 S6K, P70 S6K, GAPDH (loading control) from lysates from four patient sets. (c) Patients with FXS show increased pS473 Akt (PKB) levels in the brain compared to normal controls ($P = 0.0298$). The percent (%) of phospho-signal was normalized to total protein signal for graph. (d) Representative Western blot images for pS473 AKT and total AKT from lysates from four patient sets. (FXS, $n = 4$; *N*, $n = 4$) (e) Patients with FXS ($n = 4$) show no difference in pERK1/2 levels compared to normal controls ($n = 4$), for both pERK1 or pERK2. The percent (%) of phospho-signal normalized to total protein signal for graph. (f) Representative Western blot images for pERK 1/2 total ERK1/2 from lysates from four patient sets. (g) Levels of CYFIP2 in the frontal lobe are not different than normal controls. CYFIP2 (145 kDa band) normalized to total GAPDH protein signal for graph (FXS, $n = 4$; normal, $n = 4$). (h) Representative Western blot images for CYFIP2, GAPDH (loading control) from lysates from four patient sets. The presence of the larger band ~150 kDa was only seen in brain derived samples. All ECL signal detection was non-saturation (65K bit detection, GE Las400 imager). The error bars represent standard error in each graph. Blots were checked for efficient stripping prior to reprobing.

with FXS and normal age-matched individuals (Table 1) and isolated proteins from homogenates. Comparable to what we observed in lymphocytes, we detected a ~70% increase in the levels of phospho-T389 S6K1 in the brain (Fig. 4a) (pT389 S6K1/Tot S6K1: FXS mean = 0.333, SD = 0.067; normal mean = 0.198, SD = 0.064, df = 7, $t = 2.78$, $P = 0.0274$). The increased levels of p389 S6K1 was not due to overall difference in protein levels as GAPDH expression was indistinguishable between FXS and normal controls (Fig. 4b). Similar to what we observed in lymphocytes, we did not see a change in overall phospho-mTOR/Total mTOR (data not shown). We also examined pS473 levels in the brains of FXS patients and again, consistent with what we observed with lymphocytes, we observed increased pS473 levels in the brains of FXS individuals compared to normal controls (Fig. 4c,d) (pS473 Akt/Tot Akt: FXS mean = 0.908, SD = 0.161; normal = 0.444, SD = 0.063; df = 7, $t = 2.72$, $P = 0.03$).

We also assessed pERK 1/2 levels in brain as they have been reported to be higher in the hippocampus of the mouse models of FXS (Hou *et al*. 2006). Interestingly, pERK 1/2 levels in the frontal lobe of patients with FXS were no different than normal controls (Fig. 4e,f) ($P > 0.5$ for both pERK1/ERK1 and pERK2/ERK2). This result is not likely an artifact of post-mortem tissue treatment as pERK 1/2 differences have been reliably detected from other post-mortem samples (Dwivedi *et al*. 2006).

With respect to CYFIP2, we detected a trend toward increased expression in brain tissue from all four subjects with FXS but the difference did not reach statistical significance (Fig. 4g) (CYFIP2/GAPDH: FXS mean = 0.331, SD = 0.109; normal mean = 0.221, SD = 0.079; df = 7, $t = 1.60$, $P = 0.15$). Unlike immunostaining of the protein isolated from the blood (Fig. 3d), staining for CYFIP2 in the brain revealed two bands of closely related size, one at the predicted size (~145 kDa) and one slightly larger (Fig. 4h). Several reports have identified CYFIP2 with a single band (Jackson *et al*. 2007; Mayne *et al*. 2004; Mongroo *et al*. 2011) consistent with what we observe in lymphocytes. However, multiple bands for CYFIP2 have also been reported (Derivery *et al*. 2009). The presence of the larger band may indicate the presence of brain specific post-translationally modified CYFIP2 isoforms, expression of CYFIP2 paralogs, or the presence of peptides in the brain that are non-specifically recognized by the CYFIP2 antibody used for this study. Levels of CYFIP2 in the cerebellum were too low to quantify reliably (data not shown).

We also examined phosphorylation of eIF4E from brain tissue and found that although the total protein was easily detectable, phosphorylation levels at S209 was extremely low (data not shown). Although it is possible that pS209 signal was degraded by phosphatase activities associated with the post-mortem interval of tissue retrieval, the detectability of pT389 and pERK1/2 argue against this. This low level of frontal lobe pS209 eIF4E may in fact indicate very low steady-state eIF4E activation in the human brain. Because it is known that there are important differences in steady-state metabolic rates (Sokoloff 1981) and thus translational rates in the brain, it is possible that what we observe is specific to the frontal lobe and that other regions may display differences between FXS and normally developing individuals. Regardless, our lymphocyte data, combined with pT389-S6K1 brain results shown in Fig. 4a, is consistent with the idea that the loss of FMRP in brains of FXS patients results in dysregulation of mechanisms of translational initiation control rather than transcriptional regulation.

Discussion

Emerging evidence from both human patients with ASD and mouse model of FXS (Hagerman *et al*. 2010; Sharma *et al*. 2010) indicates that mTOR signaling cascade dysregulation and eccentric protein synthesis are present and may provide the molecular markers that could enhance diagnostics for the development of potential treatment regimens and will allow evaluation of therapies aimed at ameliorating FXS-associated symptoms. The identification of mTOR signaling dysregulation, even in a subset of patients with FXS may also open up new avenues for therapeutic intervention in pathways unrelated to translation. Thus, this study was undertaken to examine a possible role for mTOR signaling in FXS and our findings indeed implicate dysregulation of mTOR signaling, which may lead to the impaired cognitive functions observed in subjects with FXS.

Our results show a robust activation of the mTOR substrate, S6K1 at the mTOR-dependent phosphorylation site, T389, and in the phosphorylation levels of the upstream mTOR regulator, Akt at S473, in both lymphocytes and brains of subjects with FXS. Somewhat surprisingly, we did not observe a statistically significant increase in mTOR phosphorylation levels at S2448 in FXS lymphocytes, although there was a trend for increased phosphorylation at this site. These findings indicate that although the mTOR substrate, S6K1, and mTOR regulator, Akt, both show enhanced activation in lymphocytes, this enhancement does not likely extend in an S6K1-mediated feedback regulatory loop to S2448 on mTOR. It may also indicate that this mTOR phospho-site does not have the same correlational relevance as it does in other tissue and cell-culture types (Reynolds *et al*. 2002). mTOR phosphorylation is tightly regulated and changes in phosphorylation levels of greater than 50% are rarely observed even under ideal conditions (Avni *et al*. 1997). Thus, it is also possible that increased phosphorylation is indeed present but our quantitative resolution using whole lysates is not sufficient to resolve small differences in basal activation. Further, the larger sample size and much higher variance for the full mutation group compared to the normal group may have underpowered our ability to analyze results from this phosphorylation site. Finally, although phosphorylation levels at the S2448 site is correlated with increased mTOR activity (Reynolds *et al*. 2002), it is not required for it. In HEK293 cell-culture studies where mTOR Serine 2448 was replaced with an Alanine, no loss of translational efficiency was observed (Sekulic *et al*. 2000). So it may be that in lymphocytes this site is not an appropriate marker of mTOR activation.

Although we have demonstrated dysregulation of the mTOR cascade signaling, we did not detect any alteration in the ERK1/2 signaling in either lymphocytes or brain tissue from individuals with FXS. Our findings are consistent with a previous study where using subject lymphocytes, early activation kinetics of ERK were found to be different between patients with FXS and typical developing controls but steady-state (i.e. resting) levels were not (Weng *et al*. 2008). It is also possible that ERK signaling in brains of FXS patients is perturbed in region or cell-specific manner (i.e. hippocampus) and that our power to detect changes was obfuscated by the gross anatomical level of dissection used for lysate preparation. A more detailed examination of ERK signaling from specific brain areas using sectioning will be needed to examine this question in greater detail.

Consistent with what we observed in an earlier study (Nowicki *et al*. 2007); we saw decreased *CYFIP1* mRNA expression levels in lymphocytes from subjects with FXS and more so in those with the PWP (Table 1). However, a similar study reported an increase in expression of *CYFIP1* mRNA (Nishimura *et al*. 2007). This study also reported dysregulation in *JAKMIP1* and *GPR155* expression from a variety of sources: lymphoblasts from dup (15q) and non-dup (15q) idiopathic ASD patients, FXS model mice, and finally in neuronal cell lines over-expressing *CYFIP1*. They found cases of both upregulation and downregulation of these genes depending on the source of the genetic manipulation. Our study differs from Nishimura *et al*. (2007) in several important ways. First, our lymphocytes were from patients specifically identified from FXS and FXS with PWP populations rather than from a much larger ASD patient pool and we measured expression

levels directly in peripheral blood leucocytes rather than in lymphoblastoid cells lines, which often do not behave as the fresh cells from which they are derived. Second, Nishimura *et al*. (2007) confirmed their *JAKMIP1* expression in *Fmr1* KO mice and neuronal cell lines by Western blot analyses rather than mRNA expression. Third, their study used primarily microarray analyses compared to RT-PCR in our study. Finally, it should be noted that earlier microarray studies using pooled FXS lymphoblastoid cells or tissue from *FMR1* KO mice did not report *CYFIP1* or *JAKMIP1* expression differences (Brown *et al*. 2001; Miyashiro *et al*. 2003). Thus, our different results may be explained by differences in sample source, microarray type, or statistical analyses used. Although *CYFIP1* mRNA levels appear to be clearly reduced in subjects with FXS and the PWP, the correspondent decreased expression level of CYFIP1 protein observed was based on a small sample size analyzed; thus, future studies are required to investigate this possibility and determine if the difference in protein expression in this subgroup of individuals with FXS, is statistically significant.

In contrast to what we observed for *CYFIP1*, we saw no difference in *CYFIP2* mRNA expression but rather an increase in CYFIP2 protein levels between FXS, FXS with the PWP and normal control groups (Table 1, Fig. 3). *CYFIP2* is located at 5q33.3 and highly homologous to *CYFIP1* and like CYFIP1 binds FMRP (Schenck *et al*. 2003). Interestingly, *CYFIP2* mRNA contains no obvious structure that would be recognized by FMRP (Levanon *et al*. 2005) although it was identified in a screen aimed at isolating mRNPs associated with FMRP (Darnell *et al*. 2011). It is possible that RNA secondary structure prediction has important limitations with respect to FMRP function or that CYFIP2 interaction with FMRP mRNPs occurs through the activities of alternative RNA-binding proteins that interact with FMRP. Two obvious possibilities are fragile X related protein 1 (FXRP1) and fragile X related protein 2 (FXRP2), which have been shown to interact with CYFIP2 but not CYFIP1 (Napoli *et al*. 2008). Our finding of increased expression levels of CYFIP2 in FXS is potentially significant in the context of CYFIP2 relationship to apoptosis (Jackson *et al*. 2007; Saller *et al*. 1999). CYFIP2 is an p53-inducible target that may be pro-apoptotic (Jackson *et al*. 2007), thus it is possible that some symptoms of FXS are mediated by perturbation of apoptotic/cell death. In support of this idea are the aging problems that are associated with FXS including Parkinson symptoms, cognitive decline and MRI changes (Utari *et al*. 2010). Further examination of CYFIP2 protein expression in tissue of patients with FXS or FXS model mice will be required to address this important question.

Dysregulation of CYFIP1/2 levels at either the protein or mRNA level also have the potential to influence the activities of actin enucleating WASP family verprolin homologous protein (WAVE) complex (Derivery & Gautreau 2010; Takenawa & Suetsugu 2007). The multi-protein WAVE complex is composed five core subunits, which include either CYFIP1 (also known as Sra1) or CYFIP2 (also known as Pir121) (Derivery *et al*. 2009; Takenawa & Suetsugu 2007). The WAVE complex is critically involved in cell motility and lamellipodium formation and has been shown to play an important role in axon guidance and thus the development of the nervous system (Schenck *et al*. 2003, 2004; Schrander-Stumpel *et al*. 1994; Suetsugu *et al*. 2003; Tahirovic *et al*. 2010). Abnormal dendritic spine morphology and maturation are observed in the brains of FXS patients and in FXS model mice (Comery *et al*. 1997; Irwin *et al*. 2000). Because these processes critically rely on actin cytoskeletal network functions, it is possible that these FXS-associated cellular phenotypes arise from altered WAVE activity resulting from increased FMRP-mediated CYFIP2 expression. This interesting possibility can be investigated in future studies using mouse FXS models and neuronal cell culture where WAVE complex components can be experimentally manipulated and dendritic spine morphology examined along a developmental time–course.

Our biochemical data can clearly distinguish full mutation patients with FXS from normal controls. We were, however, unable to further discriminate FXS with and without PWP using molecular markers of translation initiation. Finding that mTOR signaling is dysregulated in patients with FXS (or a subset) may help explain the wide degree of clinical severity presented by FXS. More importantly, the availability of such diagnostic tools may provide insight into the therapeutic course one should take in treating individuals with FXS. Finally, molecular markers of mTOR signaling may also provide outcome measures as a means to assess the long- and short-term therapeutic efficacy of pharmaceutical interventions being used to treat FXS. Additional studies will be needed to better understand the relationship between the loss of FMRP and translational dysregulation in FXS.

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Research report

Pharmacological treatment of fragile X syndrome with GABAergic drugs in a knockout mouse model

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ABSTRACT

Molecular and electrophysiological studies have provided evidence for a general downregulation of the GABAergic system in the *Fmr1* knockout mouse. $GABA_A$ receptors are the main inhibitory receptors in the brain and the $GABA_A$ receptor was proposed as a novel target for treatment of the fragile X syndrome, the most frequent form of intellectual disability. This study examined the functionality of the $GABA_A$ receptor in rotarod and elevated plus maze tests with fragile X mice treated with $GABA_A$ receptor agonists, the benzodiazepine diazepam and the neuroactive steroid alphaxalone. In addition, the effect of $GABA_A$ receptor activation on the audiogenic seizure activity was determined. We proved that the $GABA_A$ receptor is still sensitive to GABAergic drugs as the sedative effect of diazepam resulted in a decreased latency time on the rotarod and alphaxalone had a clear anxiolytic effect in the elevated plus maze, decreasing the frequency of entries, the total time spent and the path length in the closed arms. We also observed that treatment with ganaxolone could rescue audiogenic seizures in *Fmr1* knockout mice. These findings support the hypothesis that the $GABA_A$ receptor is a potential therapeutic target for fragile X syndrome.

1. Introduction

Fragile X syndrome is the most common form of intellectual disability. Besides cognitive impairment, patients suffer from several behavioural problems including hyperactivity, sleep problems and autistic-like behaviour [1]. Epileptic seizures are also commonly observed in patients [2]. The syndrome is caused by a dynamic expansion of a CGG triplet located within the 5′ untranslated region of the *fragile X mental retardation 1* (*FMR1*) gene [3]. Due to the dynamic mutation, the CGG repeat and the surrounding CpG island located in the promoter region of the gene become hypermethylated, leading to transcriptional silencing of *FMR1* and consequently absence of the *FMR1* protein product, FMRP [4]. FMRP is an RNA-binding protein that interacts with various neuronal mRNAs and is involved in the regulation of mRNA translation, transport and stability [5–9]. Absence of FMRP might lead to deregulation of many neuronal mRNAs eventually cumulating in the fragile X phenotype.

One of the main pathways affected in the fragile X syndrome is the $GABA_A$ receptor pathway. We have found an altered expression of several components of the GABAergic system in the *Fmr1* knockout mouse, including 8 subunits of the $GABA_A$ receptor ($\alpha_{1,\ 3\ \text{and}\ 4}$, $\beta_{1\ \text{and}\ 2}$, $\gamma_{1\ \text{and}\ 2}$ and δ), proteins and enzymes involved in synthesis (*Gad 1*), transport (*Gat 1* and *Gat 4*) and degradation (*Ssadh*) of GABA and in the clustering and targeting of the $GABA_A$ receptors at the post-synaptic membrane (*Gephyrin*) [10,11]. Underexpression was also found for all three $GABA_A$ receptor subunits (*Grd*, *Rdl* and *Lcch3*) in the fragile X fly [10]. Other groups demonstrated decreased protein levels of several $GABA_A$ receptor subunits and abnormal GABA-mediated transmission in the fragile X mouse [12–15]. The combination of all these molecular and electrophysiological findings together with the fact that $GABA_A$ receptors are implicated in anxiety, depression, learning and memory, epilepsy and insomnia, all presenting in the fragile X syndrome, led us to propose the $GABA_A$ receptor as a novel target for treatment of the fragile X syndrome [16].

As the GABAergic system is compromised in the fragile X syndrome, it cannot *a priori* be excluded that the sensitivity of the receptor for $GABA_A$ receptor agonists is reduced in fragile X patients. In order to investigate the therapeutic potential of this type of drugs, we first wanted to investigate whether the $GABA_A$ receptors in the knockout mouse are amendable to treatment. By administering equal doses of GABAergic drugs to *Fmr1* knockout

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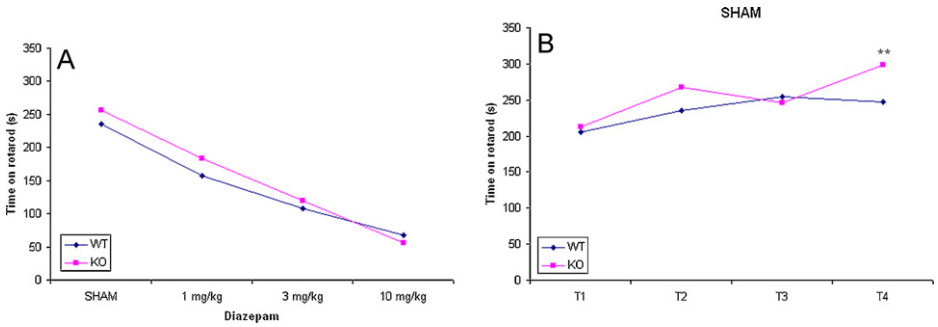

Fig. 1. Motor-coordination was tested in the rotarod assay. Time spent on the rotarod is presented for wild-type (n = 15–17/dose) and *Fmr1* knockout mice (n = 15/dose). (A) Mean total effect of diazepam on rotarod performance per concentration diazepam. (B) Performance over all trials of SHAM-treated mice. $^{**}p < 0.01$.

and control animals and comparing the performance in selected tests, we can determine the potential difference in drug sensitivity between both genotypes.

For our experiments, we selected two types of drugs that act on two different prevalent subtypes of the receptor. Diazepam, a classical benzodiazepine, binds $GABA_A$ receptors containing a β, γ_2 and either an α_1, α_2, α_3 or α_5 subunit [17]. Diazepam enhances the affinity of this most frequently present subtype of the receptor for GABA, resulting in an increased inhibition and thus a sedative effect. Neuroactive steroids are an entirely different class of agonists. Examples are the endogenous neurosteroid allopregnanolone, a metabolite of the steroid hormone progesterone and the synthetic drugs alphaxalone and ganaxolone [18]. These compounds bind predominantly to the δ-containing extrasynaptic $GABA_A$ receptor subtypes and regulate anxiety, stress and neuronal excitability by increasing both channel-open frequency and open duration [19,20]. At high concentrations, neurosteroids can even directly activate $GABA_A$ receptor channels [21]. Ganaxolone has a similar pharmacological activity as alphaxalone but due to its 3β-methyl substituent, ganaxolone is orally active and lacks hormonal side effects [22]. It was especially developed for its improved bioavailability and potential anxiolytic and anticonvulsant activity.

As sedation and anxiety are modulated through the $GABA_A$ receptor, we performed a motor-coordination test (rotarod) and an anxiety-related test (elevated plus maze) to determine the functionality of the $GABA_A$ receptor in fragile X mice. With a rotarod test, the motor-coordination and balance of a mouse is tested by placing the mouse on a rotating rod with accelerating speed. The elevated plus maze is commonly used to assess anxiety-like behaviour in mice models. The task is based on the naturalistic conflict between the tendency of mice to explore a novel environment and the aversive properties of a brightly lit, open area [23]. When anxious, the natural tendency of rodents is to prefer enclosed dark spaces over open brightly lit spaces. In addition, as several studies have shown that the $GABA_A$ receptor is implicated in epilepsy [24], we investigated the effect of GABAergic drugs on the audiogenic seizure phenotype. We did find that the $GABA_A$ receptor is a suitable target for treatment of at least some behavioural symptoms of the fragile X syndrome.

2. Materials and methods

2.1. Animals

Male *Fmr1* knockout mice and their control littermates (C57BL/6J background) were housed, bred and genotyped as described previously [10]. All experiments were carried out in compliance to the European Community Council Directive (86/609/EEC) and approved by the Animal Ethics Committee of the University of Antwerp.

2.2. Rotarod

We used an automated accelerating rotarod (Ugo Basile, Comerio, Varese, Italy; accelerating model 7650 for mice). Mice, 10 weeks old, were placed on a rotating drum and the latency to fall from the rotarod was recorded. Mice were given two practice trials (4 revolutions per minute (rpm), max 2 min) and four accelerated test trials (4–40 rpm, max 5 min), with 1 min between each trial. Mice were injected with diazepam (Roche, 1 mg/kg, 3 mg/kg or 10 mg/kg) or PBS (SHAM), 30 min prior to the test. Each group contained 15–17 mice. The results of the rotarod were analysed with a two-way ANOVA and a two-way repeated measures ANOVA.

2.3. Elevated plus maze

The elevated plus maze (EPM) is a cross-shaped maze, with two open arms and two closed arms, about a metre above the floor. It was constructed as described [23]. A stock solution of alphaxalone was made in a 22.5% 2-hydroxypropyl-β-cyclodextrin (HBC, Sigma–Aldrich; cat# 56332) aqueous solution. The solubility of lipophilic drugs increases linearly with the concentration of HBC and the product is non-toxic in rabbits and mice. AP (Steraloids Inc. Ltd., London, UK; cat# P3500-000) or an equal amount of solvent (SHAM) was injected 10 min before testing (n = 16/group for wild-type mice, n = 10–15/group for knockout mice; 15 mg/kg, i.p.).

At the start of the 10-min observation (EthoVision 3.1; Noldus Information Technology, Wageningen, The Netherlands), mice (10 weeks old) were placed on the central platform, facing one of the closed arms (preferably left). The parameters measured in the test were total path length, velocity and rearing in total EPM and frequency, time spent, path length, velocity and rearing in different parts of the EPM. Data were analysed using a two-way ANOVA and individual groups were compared by using a post hoc t-test or a Mann–Whitney rank sum test.

2.4. Audiogenic seizures

Mice were tested in an empty, transparent plastic box (28 cm × 18 cm × 17 cm) covered by a plate with a fire siren mounted on it. The box was placed into a sound-attenuating chamber equipped with a glass door for observation and the tests were recorded. After a 2-min habituation period, animals were exposed to a 120 dB noise (fire siren) until seizures were initiated or with a maximum of 5 min. Mice were tested between 02:00 p.m. and 05:00 p.m. for possible circadian variation.

Seizures were scored by the time of occurrence (test day) and by severity: no response = 0, wild running = 1, clonic seizures = 2, tonic seizures = 3 and respiratory arrest = 4. An intraperitoneal injection of diazepam (3 mg/kg) or ganaxolone (10 mg/kg) or an equal volume of vehicle (22.2% 2-hydroxypropyl-β-cyclodextrin) was administered 10 min (ganaxolone) or 30 min (diazepam and SHAM) before seizure testing. Mice were tested on 3 days with 21, 24 and 25 days of age and were injected on day 2 and 3 with drug or vehicle. Numbers of mice in each group were as follows: n = 11, 9, 12 wild-type mice and n = 18, 12, 11 knockout mice for SHAM, diazepam and ganaxolone treatment, respectively. Fisher's exact test (two-tailed) was used to analyse the percentage of seizures.

3. Results

3.1. $GABA_A$ receptors in fragile X mice are still sensitive to benzodiazepines

To investigate potential differences in the drug sensitivity of the most common $GABA_A$ receptor subtypes, we performed a rotarod test where we compared the performance of fragile X mice with control littermates using different concentrations of diazepam. We found a significant effect for treatment for both

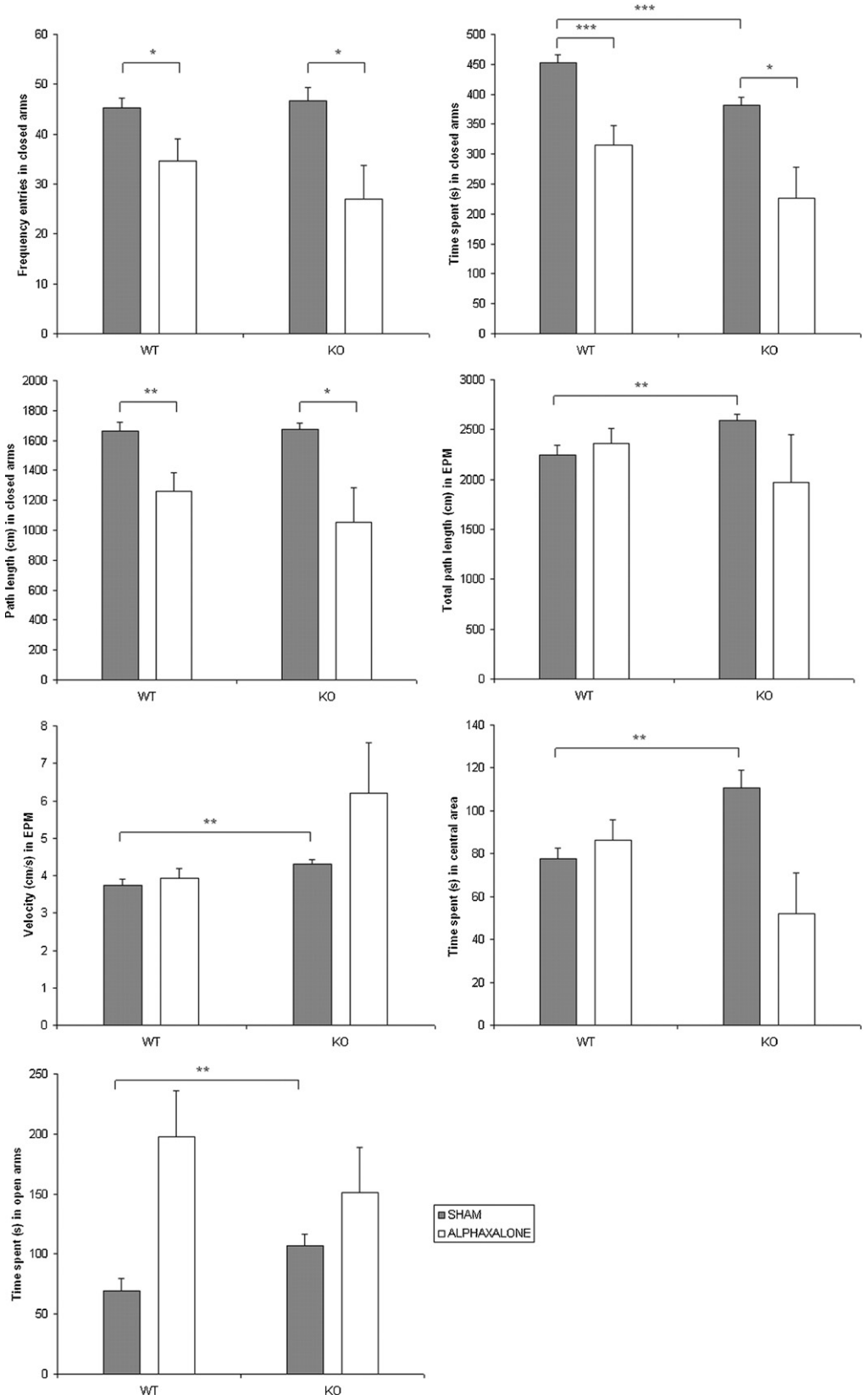

Fig. 2. Elevated plus maze. *Fmr1* knockout mice and control littermates were treated with alphaxalone or SHAM. Parameters analysed are frequency entries, time spent and path length in closed arms, total path length and velocity in total elevated plus maze, time spent in central area and time spent in open arms. Error bars indicate SEM (n = 10–16 mice/group), $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

genotypes ($F_{(3,488)} = 153.412$, $p < 0.001$, two-way ANOVA) (Fig. 1A). Wild-type mice as well as fragile X mice became more sedated with increasing doses of diazepam, as shown with the decreased latency time spent on the rotarod. This sedative effect was not significantly different between wild-type and knockout mice for all given doses of diazepam (1 mg/kg, 3 mg/kg, 10 mg/kg) (main effect of genotype $F_{(1,488)} = 3.548$, $p = 0.060$ and interaction between treatment and genotype $F_{(3,488)} = 1.655$, $p = 0.176$). Overall, SHAM-treated wild-type and knockout mice did not significantly differ in their performance over the 4 trials (interaction between genotype and trial $F_{(3,90)} = 1.989$, $p = 0.121$ and main effect of genotype $F_{(1,30)} = 1.258$, $p = 0.271$, measured with the two-way repeated measures ANOVA) (Fig. 1B). However, in the last trial, knockout mice performed better than wild-type mice ($p = 0.007$).

3.2. $GABA_A$ receptors in fragile X mice are still sensitive to neurosteroids

To investigate potential differences in the drug sensitivity of the extrasynaptic $GABA_A$ receptors, we treated fragile X mice and control littermates with the neuroactive steroid alphaxalone and compared the behaviour in the elevated plus maze between both genotypes. We were particularly interested in the entries, total time spent and path length of the mice in the closed arms, as the tendency of mice to enter or remain in the closed arms of the maze is decreased by anxiolytic drugs. Both fragile X mice and wild-type littermates showed a significant reduction in frequency of entries, in total time spent and in path length in the closed arms after treatment with alphaxalone (Fig. 2). A two-way ANOVA showed that the effect of treatment was significant in all three cases ($F_{(1,53)} = 15.327$; $F_{(1,52)} = 27.510$; $F_{(1,52)} = 19.168$; $p < 0.001$), but that the effect of genotype on the treatment was not significant ($F_{(1,53)} = 1.405$, $p = 0.241$; $F_{(1,52)} = 0.109$, $p = 0.743$; $F_{(1,52)} = 0.852$, $p = 0.360$ respectively). This suggests that the drug has a clear anxiolytic effect in both knockout and wild-type animals but that there is no difference in reaction of both genotypes to the drug. Both genotypes did not differ in most aspects measured, but some parameters measuring hyperactivity and anxiety such as the total path length ($p = 0.008$) and velocity ($p = 0.008$) in the total maze and the time spent in the central area ($p = 0.002$) and in the open arms ($p = 0.013$), were significantly higher for SHAM-treated knockout mice than SHAM-treated wild-type mice (Fig. 2).

3.3. Drugs interacting with the $GABA_A$ receptor can rescue audiogenic seizures

As we proved that the $GABA_A$ receptor is still functional in the fragile X mice, we wanted to investigate whether drugs acting on the $GABA_A$ receptor could ameliorate or rescue symptoms of the fragile X syndrome. Therefore, we performed audiogenic seizures (AGS) tests. In total, 50% of the knockout mice tested had an epileptic seizure. They reached wild-running, tonic seizures or respiratory arrest as seizure end-point scores (Fig. 3). No audiogenic seizures were observed in wild-type mice. As expected, after injection of the well-established anti-epileptic drug diazepam, no epileptic seizures could be provoked anymore ($p = 0.024$). In addition, the neuroactive steroid ganaxolone was also able to abolish the epileptic seizures; in fact, no mouse exhibited any seizure response ($p = 0.026$). This indicates that both benzodiazepines as well as neuroactive steroids are able to rescue this particular fragile X phenotype.

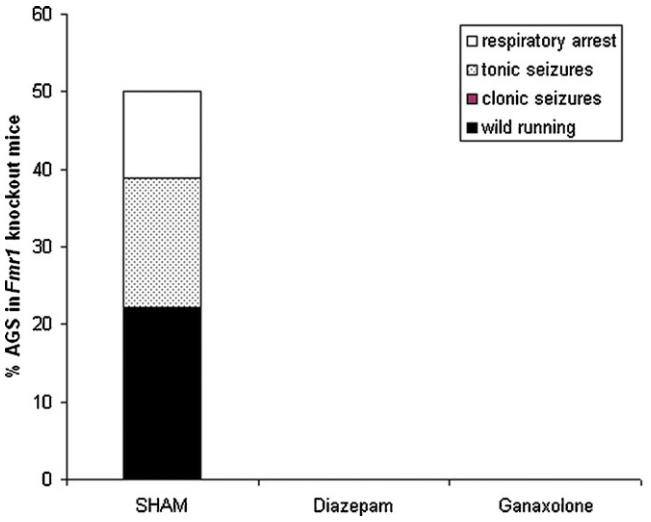

Fig. 3. Audiogenic seizure (AGS) incidence in *Fmr1* knockout mice after SHAM, diazepam and ganaxolone treatment ($n = 11$–18 mice/group).

4. Discussion

In this study we investigated the functional potential of the $GABA_A$ receptor as a novel target for treatment of the fragile X syndrome. In the first place, it is of utmost importance that the receptor still responds to $GABA_A$ receptor agonists. The functionality of the $GABA_A$ receptor was demonstrated in fragile X mice in two behavioural tests, a motor-coordination test and an anxiety-related test.

Motor-coordination was tested with an accelerating rotarod. Overall, we did not find a significant difference between the performance of wild-type and knockout mice at SHAM treatment. This is in line with previous observations where the performance of knockout and wild-type mice was similar during the early trials [25]. When a more-day training paradigm was executed, knockout mice became mildly impaired in comparison with wild-type mice [25–27]. As our experimental set-up did not include a prolonged period of testing, we could not observe this impairment in motor-coordination or determine a real motor learning effect. However, we rather observed a trend of knockout mice performing equally or even better than wild-type mice in the later trials.

Previous measurements of anxiety in the fragile X mouse model have given inconsistent results. In most studies fragile X mice seem to be less anxious than wild-type mice, but in other anxiety-related tests, such as the open field and light–dark exploration test, no differences or even increased anxiety was observed [25,26,28–36]. Inconsistent results were also observed in the elevated plus maze, which is considered the most robust tests for measuring anxiety [29,33,37]. Differences between test outcomes can be caused by the genetic background, the age of the mice but also by test and environmental conditions [38]. Our protocol shows most resemblance with that used by Yuskaitis et al. [33] and our results most closely resemble their observations. In our test, knockout mice are less anxious as they spent significantly more time in the open arms than wild-type mice. We could not formally detect hyperactive behaviour for knockout mice as the total number of entries, a built-in control for general hyperactivity, was not higher than for wild-type mice. However, the total distance travelled, as well as the velocity in the elevated plus maze was significantly higher for knockout mice.

In a third behavioural assay, we were able to completely rescue the audiogenic seizures in knockout mice using diazepam and the neuroactive steroid ganaxolone. The efficacy of diazepam was already demonstrated in earlier studies showing that diazepam reduces audiogenic seizures in DBA/2 mice, and both chemical- and electroshock-induced seizures in C57BL/6 mice [39–41]. Ganaxolone has shown its anticonvulsant effects in diverse animal models

and has shown its efficacy in the treatment of partial seizures, infantile spasms and catamenial epilepsy [18,42]. However, this is the first time that the efficacy of ganaxolone in the treatment of audiogenic seizures in the fragile X syndrome is demonstrated.

Taken together, these data demonstrate that the $GABA_A$ receptor is still sensitive to GABAergic drugs and, more importantly, neuroactive steroids may be used for targeted therapy of epileptic seizures associated with fragile X syndrome. This means that despite the reduced amount of $GABA_A$ receptors in fragile X mice, they remain sensitive to benzodiazepines and neurosteroids. In addition, we provided the first pharmacological evidence that stimulating the $GABA_A$ receptor with GABAergic agonists can rescue specific behavioural symptoms of the fragile X syndrome. This is in line with results obtained in the fragile X fly. In this model, it was shown that administration of GABA could rescue most of its symptoms, including courtship behaviour and the morphological aberrations of the mushroom bodies [43]. Thus, our study encourages clinical trials in fragile X patients with drugs that target the GABAergic system. Ganaxolone is currently in phase II clinical trials for the treatment of several forms of epilepsy [18,44]. It is orally active, interactions with other anti-epileptic drugs have not yet been revealed and anticonvulsant tolerance does not develop following chronic therapy. It is well tolerated in adults as well as in children, with limited side effects. In addition, as alphaxalone was also able to reduce anxiety in fragile X mice and controls alike in the elevated plus maze, neurosteroids might also be used for the treatment of anxiety and perhaps additional behavioural abnormalities.

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